# Inter- and Intramolecular Interactions of α-Lactalbumin. II. Aggregation Reactions at Acid *p*H*





α-Lactalbumin in acid *p*H exhibits a time-dependent aggregation, the characteristics of which have been studied using sedimentation and solubility methods. Exposure of the protein to *p*H values below the region of its isoelectric point yields a component having a sedimentation constant of 10–14 S (infinite dilution) in contrast with the value of 1.93 S obtained for the monomeric molecule. The *rate* of formation of the heavy component decreases with decreasing temperature and decreasing *p*H and ionic strength. Similarly, reduction of the net molecular charge through binding of anions such as nitrate dramatically increases the rate of aggregation. In contrast with its behavior on the acid side, aggregation on the alkaline side of the isoelectric zone is almost entirely absent, only small amounts of heavy component being observed at very high protein concentrations. In fact, aggregation occurring at acid *p*H values can be completely reversed by adjusting such solutions or gels to *p*H values alkaline to the isoelectric zone. It has been shown that aggregates formed at acid *p*H have decreased solubility in solutions of ammonium sulfate at *p*H 6.6. Differences in the rates of reversal of insolubilization indicate that the acid-aggregate and the isoelectric precipitate are not identical. A study of the *p*H dependence of the aggregation showed a *decrease* in rate above *p*H 3.75, contrary to what might be expected from electrostatic considerations alone. The most likely explanation of the aggregation phenomena is that at *p*H values below 4, α-lactalbumin exists in a denatured state having lower solubility than that of the native protein. The increase in frictional ratio observed for the monomeric molecule at *p*H 2.00 as compared to *p*H 8.55 indicates a difference in molecular state at these two *p*H values. Changes are cited in differential ultraviolet spectra, rotatory dispersion, and tryptophan fluorescence, which lend support to the hypothesis that the increased tendency of α-lactalbumin to aggregate below its isoelectric region is the consequence of a "denaturationlike" process.

In the previous paper in this series (Kronman and Andreotti, 1964) we showed that the apparent heterogeneity of α-lactalbumin at *p*H values acid to the isoelectric zone was due to association and aggregation of the protein. The aggregation, which was found to be a time-dependent process, was essentially absent on the alkaline side of the isoelectric zone. In this paper we shall describe the characteristics of the acid *p*H aggregation and consider its relationship to the association process and to the conformational change occurring in this *p*H region.

## Materials and Methods

With the exception of the procedures cited below for solubility measurements, the experimental details have been described (Kronman and Andreotti, 1964).

### *Solubility Experiments*

*Preparation of Stock Solutions.*—Solutions were prepared from the ammonium sulfate slurry by dialysis versus large excesses of 0.15 M KCl for a period of 20–24 hours at 2°. Subsequent to dialysis an aliquot was analyzed for protein concentration and dilutions with 0.15 M KCl were made where required.

*p*H *Adjustment.*—The desired *p*H was attained by careful addition of very small increments of 1 N HCl with an ultramicro buret with efficient magnetic stirring. The temperature at which the *p*H was adjusted was that at which the experiment was to be carried out. The time required for adjustment was no greater than 2 minutes. The solutions were then equilibrated at the desired temperature for appropriate times, whereupon aliquots were removed for tests of solubility in ammonium sulfate, solubility at the *p*H in question, and reversal of insolubilization.

*Solubility in Ammonium Sulfate.*—To an aliquot of protein solutions was added predetermined amounts of *p*H 8.0 and *p*H 6.6 phosphate buffers to yield a protein solution at *p*H 6.6 ± 0.05. The ionic strength at this point was 0.23 in phosphate and 0.13 in KCl. *Immediately* after buffering to 6.6, saturated ammonium sulfate in *p*H 6.6, 0.15 ionic strength phosphate buffer was quickly added to yield a solution of the following ionic composition: ammonium sulfate, 2.1 M; phosphate, 0.19 ionic strength; KCl, 0.06 M. During the addition of ammonium sulfate vigorous mixing was carried out with a vortex mixer. The resulting suspension of protein was equilibrated at 25.0° for 30 minutes, whereupon the precipitate was separated by centrifugation at 25°. The decanted supernatant solution was analyzed for protein concentration.

*Reversal of Insolubilization.*—This procedure is identical with that just described for solubility in ammonium sulfate except in the manner of equilibration of the *p*H 6.6 buffered protein solution. In the former method saturated ammonium sulfate was added *immediately* after buffering to 6.6. In the reversal experiments the *p*H 6.6 protein solution was equilibrated at 25.0° for varying times before addition of the ammonium sulfate. Succeeding steps were identical with those of the previous procedure.

*Solubility Measurements.*—An aliquot of the solution or well-mixed suspension at a given *p*H and temperature was centrifuged in a centrifuge regulated at

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TABLE I

EFFECT OF ELECTROLYTE SPECIES ON α-LACTALBUMIN AGGREGATION[a]

| pH | Electrolyte | $s_{20}$ Light | $s_{20}$ Heavy | Per Cent Fast |
|---|---|---|---|---|
| 3.00 | KCl | 2.25 | | |
| 3.00 | Formate | 1.79 | 11.8 | 50 |
| 3.00 | $KNO_3$ | | Insoluble | |
| 3.00 | $(NH_4)_2SO_4$ | | Insoluble | |
| 2.00 | $KH_2PO_4$-$H_3PO_4$ | 1.90 | | |
| 2.00 | KCl | 1.95 | | |
| 2.00 | KBr | 2.09 | | Trace |
| 2.00 | $K_2SO_4$ | 2.44 | 13.3 | 26.9 |
| 2.00 | $(NH_4)_2SO_4$ | 2.55 | 12.7 | 32.3 |
| 2.00 | 0.10 M KCl, 0.05 M $KNO_3$ | 2.02 | 12.2 | 11.5 |
| 2.00 | $KNO_3$ | 2.27 | 18.1 | 59.6 |
| 2.00 | KCNS | | Insoluble in acid region | |

[a] Protein concn 1.0–1.25%; Γ/2 = 0.15; preparation no. 2-69; temp 25.0°; time of aggregation 1 hour.

the temperature of the experiment. The supernatant solution was decanted and analyzed for protein concentration.

## RESULTS

*Aggregation at Acid pH.*—The ultracentrifuge patterns presented previously (Fig. 2, Kronman and Andreotti, 1964) are typical of those observed for α-lactalbumin at *p*H values acid to the isoelectric zone. The rate of polymerization of monomeric units (rate of formation of the heavy component) depends upon *p*H, protein concentration, and temperature. This is illustrated in Figure 1 which presents data for the per cent aggregation as a function of total protein concentration at 10 and 25°. (The per cent aggregation was computed from the relative areas of heavy and light components. For very high degrees of aggregation only the area of the light component can be readily obtained. In those cases, the per cent fast, taken to be the total area minus the area of the light component, represents heavy component plus any heavy material sedimenting to the bottom of the cell. In the case of more limited aggregation, *ca.* 70% or less, the area of both components could be obtained. In general, the total area of both peaks accounted for about 95% of that anticipated for a given protein concentration.) It is quite apparent that the aggregation reaction is not an equilibrium process but is time dependent, the extent of reaction at any time being markedly dependent on concentration, particularly at 25°. The results indicate that all or nearly all of the α-lactalbumin is capable of being incorporated into "fast" component.

Even at *p*H 2.00, where the net positive charge on the α-lactalbumin is approaching its maximum value, aggregation still persists. This is illustrated by the data of Figure 1a. Comparison of the per cent fast at *p*H 2.00 with that at *p*H 3.00 (Fig. 1b) indicates that the *rate* of formation of aggregate is slower at *p*H 2.00, as one might expect on the basis of electrostatic considerations alone. Nonetheless, the marked extent to which the process has occurred at *p*H 2.00 at high concentration of protein suggests that the inherent ability of α-lactalbumin to aggregate is independent of *p*H in the acid region. This is further brought out by studies of the effect of various anions on aggregation and changes in solubility in acid *p*H which will be discussed subsequently in this paper.

*Effect of Electrolyte on Aggregation.*—α-Lactalbumin shows an extraordinary sensitivity to substitution of various anions for chloride ion. Zittle (1956) has also

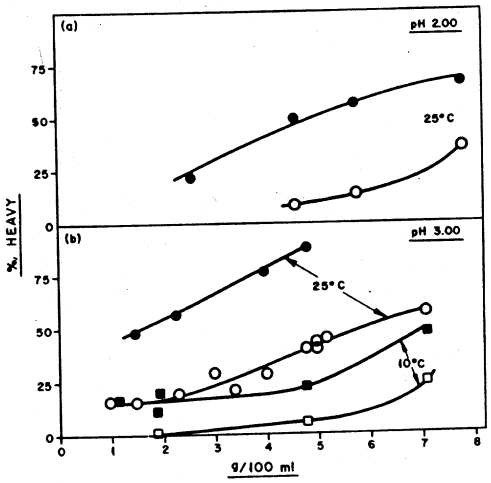


FIG. 1.—Extent of aggregation of α-lactalbumin as a function of protein concentration in 0.15 M KCl. (a) *p*H 2.00; (b) *p*H 3.00. Filled symbols, 24 hours; unfilled symbols, 1 hour. Preparation no. 3088.

noted the dependence of α-lactalbumin properties on ion binding. Shown in Table I are ultracentrifuge results obtained with a variety of anions at *p*H 2.00 and 3.00. These were obtained at 25° after 1 hour of equilibration. At *p*H 3.00 nitrate and sulfate actually precipitated the protein, while formate produced extensive aggregation. Under these conditions in 0.15 M KCl, less than 2–3% of heavy material is observed.

At *p*H 2.00 all solutions were clear with perhaps some enhanced light scattering with the exception of those containing KCNS. Apparently, in KCNS aggregation proceeds to the point of insolubility. For the other ions the ultracentrifuge revealed, however, the presence of a heavy component. The marked effect of nitrate ion is particularly noteworthy; under conditions where α-lactalbumin in 0.15 M KCl yielded no heavy component, the same concentration of $KNO_3$ produced 60% of heavy material. Even at a concentration of 0.05, nitrate ion produces a significant amount of aggregate. The sequence of effectiveness in promoting aggregation appears to be: $Cl^- = H_2PO_4^- < Br^- < NO_3^- < CNS^-$ with sulfate ion on an equimolar basis probably more effective than nitrate ion. This sequence of aggregation promotion by anions is essentially the sequence of binding that Carr (1952) and Scatchard and Black (1949) have found for anions and bovine serum albumin. If we assume that the sequence for α-lactalbumin is the same as for serum albumin, we

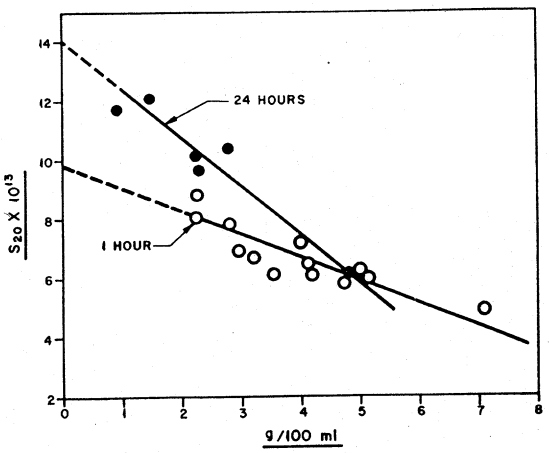


FIG. 2.—Concentration dependence of the sedimentation constant of the heavy component. *p*H 3.00; 0.15 M KCl; 25°; O, 1-hour exposure; ●, 24-hour exposure.

are led to conclude that the sensitivity of the aggregation process to the nature of the supporting electrolyte is a consequence of the reduction of the net charge on the protein molecule due to anion binding.

This latter conclusion finds support in the observation of the effect of ionic strength upon aggregation. As the data of Table II indicate the amount of heavy component seen in the ultracentrifuge after 24 hours of aging of solution is markedly decreased as we reduce the ionic strength from 0.15 to 0.02. Apparently, as the screening effect of the electrolyte is reduced the electrostatic energy barrier to aggregation is greatly increased with a concomitant reduction of aggregation rate.

TABLE II
EFFECT OF IONIC STRENGTH ON AGGREGATION AT *p*H 3.00[a]

| | % Heavy | |
|---|---|---|
| I/2 | 1 hour | 24 hours |
| 0.02 | <5 | 11 |
| 0.05 | 7 | 44 |
| 0.15 | 30 | 82 |

[a] Preparation 45G; 25.0°; protein concentration, 4.10–4.17 g/100 ml; salt, KCl.

*Effect of Method of Preparation on Aggregation.*—α-Lactalbumin prepared by the procedures of Aschaffenburg and Drewry (1957), of Gordon and Semmett (1953), and of Robbins and Kronman (1964) all aggregated to essentially the same extent under comparable conditions at acid *p*H. Since the two latter preparative methods do not involve the *p*H 2 salting-out inherent in the former, it is apparent that the time-dependent aggregation is not the consequence of irreversible changes induced during preparation. This is in contrast with the observations of Cann and Phelps (1957), who found for conalbumin that ammonium sulfate precipitation at acid *p*H resulted in subtle but rather significant changes in its aggregation properties.

*Aggregate Size.*—From the sedimentation constants of the heavy component one can make crude estimates of the "molecular" size of the sedimenting unit. We are assuming for the sake of this discussion that the heavy component represents a single species. Shown in Figure 2 is a plot of the sedimentation constant of the heavy component as a function of concentration for aggregate formed after 1 hour and after 24 hours. These data were obtained in the experiments summarized in Figure 1. The sedimentation constants for these two times of exposure to acid *p*H fall on different curves, the data at 1 hour extrapolating to about 10 S while those for 24 hours tend toward 14 S. It is thus apparent that a unique particle size is not characteristic of the process. This conclusion is lent support by the observation (Table I) that the aggregate obtained in the presence of 0.15 M potassium nitrate is about 50% higher than that obtained in 0.15 M potassium chloride at comparable protein concentration (Fig. 2).

If one were to assume the aggregate to be a compact sphere, the "molecular" weights would be of the order of 150,000–300,000 (10 and 14 S). These values clearly represent lower limits to the size since the assumption of a spherical shape is probably a rather crude assumption. Rees (1951), for example, has pointed out that aggregates of charged molecules like α-lactalbumin at acid *p*H tend to be asymmetric.

*Reversibility of the Aggregation.*—For a process as gross as the aggregation of α-lactalbumin in acid solution, a question arising immediately is, can it be reversed? Experiments carried out in the acid and alkaline sides of the isoelectric point show that it can.

REVERSAL IN ALKALINE MEDIUM.—Since experiments discussed previously indicated that *p*H 8.55 α-lactalbumin exists in monomeric form, this *p*H was chosen for the reversal experiments. Shown in the first column of Table III is the scheme used in these experiments. Because of the marked effects observed in nitrate ion at *p*H 2.00 (Table I), this particular system was chosen for study. As we can see from the second column of Table III, after 24 hours at *p*H 2.00 the solution in nitrate ion had set to a gel. Nonetheless, upon adjustment to *p*H 8.55 with KOH the gel dissolved completely and examination in the ultracentrifuge revealed but a single peak of sedimentation constant essentially identical with that obtained for this protein exposed to chloride ion at *p*H 2.00. Shown also in parenthesis is the value of the sedimentation constant obtained for α-lactalbumin which had not been acid exposed. Apparently neither the chloride nor the nitrate-exposed protein can be distinguished at *p*H 8.55 from the original material. As a further test of reversibility the aggregation properties of the chloride-exposed and nitrate-exposed material were compared after removal of the nitrate ion by dialysis. As we can see from Table III, the component distribution and sedimentation constants in both cases are essentially the same. It would thus appear that the acid *p*H aggregation is fully reversible.

REVERSAL AT ACID *p*H.—As a test of the reversibility of the aggregation an α-lactalbumin solution of 0.9 g/100 ml was aged for 1 hour at *p*H 2.00 in 0.15 M $KNO_3$. Examination in the ultracentrifuge revealed a component distribution much like that shown in Figure 1a, i.e., more than 60% of the protein moved as 18–20 S component. This solution, upon removal of the nitrate ion by dialysis against *p*H 2, 0.15 M KCl at 1–2°, readjustment to *p*H 2.00, and equilibration for 1 hour at 25°, gave essentially a single peak (1.8 S) with a very small amount (less than 5%) of a 4–5 S component.

On the other hand, attempts to reverse the aggregation at *p*H 3.00 in 0.15 M KCl by dilution were unsuccessful. In this experiment at 25.0° a protein solution of concentration 7.08 g/100 ml was allowed to "age" for 1 hour at *p*H 3.00. It was then diluted with *p*H 3.00, 0.15 M KCl to a concentration of 2.31 g/100 ml and immediately run in the ultracentrifuge. An aliquot of the original protein solution from which the dilution had been made was also run at the same time in a second ultracentrifuge. The component distribution

TABLE III
REVERSAL OF α-LACTALBUMIN AGGREGATION[a]

| | Per Cent Fast | | $s_{20} \times 10^{-13}$ (slow) | | Protein Concentration (g/100 ml) | |
|---|---|---|---|---|---|---|
| | 0.15 M KCl | 0.15 M $KNO_3$ | 0.15 M KCl | 0.15 M $KNO_3$ | 0.15 M KCl | 0.15 M $KNO_3$ |
| pH 2.00, 1 hr | 6 | 86 | 1.71 | 2.32 | 3.05 | 2.64 |
| pH 2.00, 24 hr | 25 | Gel | | | 3.05 | 2.64 |
| ↓ | | | | | | |
| pH 8.55 | 0 | 0 | 1.69 (1.70) | 1.71 | 2.40 | 2.61 |
| ↓ | | | | | | |
| Dialysis versus 0.15 M KCl | | | | | | |
| ↓ | | | | | | |
| pH 5.9 | | | | | | |
| ↓ | | | | | | |
| pH 2.00, 1 hr | 7 | 5 | 1.71 | 1.75 | 2.37 | 2.54 |
| pH 2.00, 24 hr | 15 | 15 | 1.72 | 1.76 | 2.37 | 2.54 |

[a] Preparation 3088; carried out at 25.0°; speed, 59,780 rpm.

of *both* the diluted *and* the original solution were essentially the same, 50–55% heavy component. As we see from Figure 2, the per cent of heavy material in a solution of concentration 2.31% should be about 20%. Apparently, during the time interval between dilution and observation (*ca.* 30 minutes before the ultracentrifuge was brought to speed), little if any reversal occurred.

*Solubility Changes at Acid pH.*—Changes with time in the state of aggregation of α-lactalbumin can be likewise demonstrated by means of solubility measurements. The procedures employed are described in detail under Materials and Methods. In essence they involve exposure of α-lactalbumin to acid *p*H for a predetermined length of time, rapid buffering to *p*H 6.6, and measurement of the extent of precipitation in the presence of 2.1 M $(NH_4)_2SO_4$. By making such measurements at identical *p*H and electrolyte composition we avoid the question of the effect of *p*H on solubility of the aggregate and are able to follow *changes* in solubility with time. This, of course, is the procedure frequently used to study the kinetics of protein denaturation.

Shown in Figure 3 are typical results obtained at *p*H 3.00. Notice that over a period of about 24 hours approximately 90% of the α-lactalbumin becomes converted to a form insoluble under the conditions of the test. This decrease in solubility can be reversed by exposure to *p*H 6.6 for varying times *prior* to addition of ammonium sulfate. The course of this reversal process for protein aggregated at *p*H 3 is likewise shown in Figure 3a. This was obtained for α-lactalbumin which had been exposed to *p*H 3.00 for 24 hours and which at that time had a solubility of about 10%. As can be seen the reversal is relatively rapid, 50% of the change occurring in 1 hour. Yet, the rate of reversal must be still slow enough that, under the condition of the normal test, reversal does not occur during the time interval between buffering and addition of ammonium sulfate (*ca.* 30 seconds). Shown also for comparison are the results of a control experiment where the original protein stock solution was buffered to *p*H 6.6 and the changes in solubility in 2.1 M ammonium sulfate were followed. It is apparent that exposure to acid *p*H produced no irreversible changes in solubility in that the original solubility was regained. (Actually it would appear that in many instances the solubility attained after reversal is *higher* than that of the stock solution. The reason for this is not clear. Perhaps association occurring in the dialyzed ammonium sulfate solution is "frozen in" and can be reversed only by initial acidification. This would seem

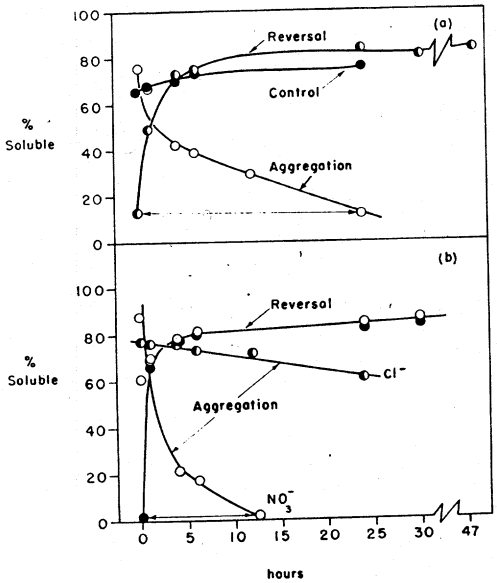


FIG. 3.—Time dependence of the solubility of acid-treated α-lactalbumin in 2.1 M ammonium sulfate at *p*H 6.6 at 25.0°. Preparation 44G. (a) *p*H 3.00, 0.15 M KCl; (b) *p*H 2.00; O, 0.15 M $KNO_3$; ●, 0.15 M KCl.

to find support in the observation [Fig. 3a,b] that the zero-time solubility in acid *p*H is often higher than that of the control solution. This point has not been considered further.)

Reversal of the aggregation process at *p*H 2.00 in the presence of 0.15 M potassium nitrate is also relatively rapid. This is shown in Figure 3b, together with the time course of insolubilization. Also shown is the reversal and insolubilization curve for protein at the same concentration of potassium chloride. The rate of insolubilization in chloride ion is considerably slower than in nitrate ion. Nonetheless, as the reversal curves indicate, the extent of recovery of the original solubility is essentially the same. Both the relative rates of insolubilization and the completeness of reversal are in accord with the ultracentrifuge studies of the aggregation in nitrate and chloride ions.

Attempts to describe the rates of insolubilization in terms of simple kinetic schemes were unsuccessful, the data showing wide deviation in either first- or second-order plots even for very short times. Qualitatively these measurements showed that the rates of insolubilization decreased with temperature and, as the data

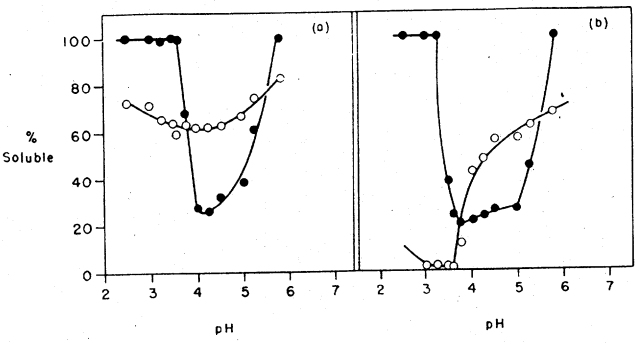


FIG. 4.—Comparison of "solubility" of α-lactalbumin with solubility in 2.1 M ammonium sulfate at 25.0°. O, "solubility" at the *p*H indicated; ●, solubility in 2.1 M ammonium sulfate at *p*H 6.6. Detailed procedures given in experimental section. Preparation 44G; (a) zero time; (b) 24 hours.

of Figure 3a,b illustrate in part, increase with increasing *p*H.

The cited characteristics of the insolubilization process are comparable to those observed for the aggregation reaction observed in the ultracentrifuge. It thus seems reasonable to identify the changes in solubility with the aggregation process observed in the ultracentrifuge. If we make this assumption, it now becomes possible to study the aggregation process at *p*H values close to and within the isoelectric region where ultracentrifuge measurements are precluded.

*Solubility in the Region of the Isoelectric Point.*—The existence of a zone of insolubility in the isoelectric region of α-lactalbumin immediately leads to the question as to a possible relationship between the acid *p*H aggregation and the isoelectric precipitation. In order to answer this, two types of measurements have been compared. The first of these is the determination of solubility of the protein at a given *p*H; the second, the solubility in 2.1 M ammonium sulfate at *p*H 6.6. The detailed procedures for both of these are given under Materials and Methods.

The data shown in Figure 4a,b were obtained using a single stock solution. Both types of solubility measurement at a given *p*H were made on aliquots of the same solution withdrawn at the same time. The filled circles represent solubilities at the *p*H indicated for zero time and 24 hours. The zero-time values were obtained immediately after *p*H adjustment. The solubility curves show the anticipated minimum in the isoelectric region (4.25–4.5) and are similar to that reported by Maeno and Kiyosawa (1962) for human α-lactalbumin, although the numerical values are quite different from theirs. The reason for this has been considered in the previous paper (Kronman and Andreotti, 1964).

The unfilled circles represent solubilities in ammonium sulfate at *p*H 6.6 of protein exposed to the *p*H indicated. The decreased values on the acid side of the isoelectric region at zero time may be a consequence of the aggregation occurring in the finite time required for *p*H adjustment. After 24 hours the ammonium sulfate solubility of α-lactalbumin shows the anticipated marked decrease in the *p*H range 2.5–3.6. What is rather surprising is the higher ammonium sulfate solubility in the *p*H region above 3.75. The previously demonstrated effect of *p*H, ionic strength, and anion species on the aggregation of α-lactalbumin in acid *p*H would lead us to believe that, in general, reduction of electrostatic repulsion among α-lactalbumin molecules would lead to enhancement of the rate of aggregation. Thus, as we go from *p*H 3.75 toward the isoelectric point (*p*H 4.25–4.5), we would predict an *increasing* rate of aggregation. This is contrary to what is observed.

Another surprising feature of these solubility curves can be seen by comparing the two types of measurements in the *p*H region 4–5. We see that the *p*H 6.6 ammonium sulfate solubility is higher at *both* zero time and 24 hours than the actual solubility at the *p*H in question. The significance of this can be seen best by casting the data of Figure 4 in another form. This is shown in Table IV. The concentrations in columns

TABLE IV
SOLUBILITY (g/100 ml) IN *p*H 6.6, 2.1 M $(NH_4)_2SO_4$

| | 0 Hours | | 24 Hours | |
|---|---|---|---|---|
| *p*H | (1) Observed | (2) Predicted | (3) Observed | (4) Predicted |
| 4.00 | 0.759 | 0.343 | 0.510 | 0.251 |
| 4.25 | 0.759 | 0.328 | 0.570 | 0.280 |
| 4.50 | 0.762 | 0.393 | 0.667 | 0.304 |
| 5.00 | 0.824 | 0.474 | 0.680 | 0.315 |
| 5.25 | 0.909 | 0.743 | 0.742 | 0.539 |

(1) and (3) were determined experimentally and are equal to

$$\frac{(\%\text{ soluble in ammonium sulfate})\ (1.22\text{ g}/100\text{ ml})}{100}$$

where 1.22 g/100 ml is the *total* concentration of protein under the conditions of the ammonium sulfate–solubility test. The predicted solubility given in columns (2) and (4) are those expected *if precipitate formed at the* pH *in question was likewise insoluble at* pH *6.6 in ammonium sulfate.* This is equal to

$$\frac{(\%\text{ soluble})\ (1.22\text{ g}/100\text{ ml})}{100}$$

where the % soluble now refers to the *solubility at the given* pH *rather than in ammonium sulfate.*

As the data of Table IV indicate the predicted values are markedly lower than the observed values in the *p*H region 4–5.25. The only reasonable explanation of this difference seems to be that *precipitate* formed in this *p*H region dissolves rapidly at *p*H 6.6 before addition of ammonium sulfate. This is in marked contrast with the behavior of aggregate formed at *p*H values below 4 where reversal at *p*H 6.6 was slow enough to permit salting out with ammonium sulfate. Thus it would appear that, on the basis of the rates of reversal at *p*H 6.6, aggregate formed below *p*H 4 differs from the isoelectric precipitate. This difference in the mode of aggregation is quite evident visually; after 24 hours of equilibration one observes successively in going from *p*H 2 toward the isoelectric point: clear solutions, turbidity, gels at *p*H 3.5 and 3.6, and finally precipitates above *p*H 3.6.

*Molecular State of Monomeric α-Lactalbumin at* pH *2.00.*—The marked differences in the aggregation and solubility behavior of α-lactalbumin on the acid and alkaline sides of the isoelectric point appear to be a reflection of differences in their molecular state (see also Kronman and Andreotti, 1964). This can be seen on comparison of their sedimentation constants at infinite dilution. Shown in Figure 5 is a plot of $s_{20}$ versus concentration for the light component at *p*H 2.00. The presence of a maximum in this curve is a reflection of the previously described association (Kronman and Andreotti, 1964).

A rather surprising feature of this curve is the trend toward low values of $s_{20}$ at low protein concentration.

Comparison of these values with the sedimentation curve obtained at *p*H 8.55 (dashed line; Kronman and Andreotti, 1964) indicates a significant decrease of the sedimentation constant below the value of 1.93 S obtained for the monomeric molecule at infinite dilution at the latter *p*H.

Direct measurement of the molecular weight at *p*H 2.00 (Fig. 5, Kronman and Andreotti, 1964) excludes the possibility of dissociation of the molecule into subunits, which also might have accounted for the low sedimentation constant. Thus the low values of sedimentation constant observed at *p*H 2.00 must be a reflection of an increase in hydrodynamic volume of the monomeric unit.

A comparison of the frictional properties of the native and the *p*H 2–exposed molecule is presented in Table V. The values of axial ratio and molecular radius

TABLE V
COMPARISON OF FRICTIONAL PROPERTIES OF NATIVE AND ACIDIFIED α-LACTALBUMIN

| *p*H | $s_{20} \times 10^{13}$ | Frictional Ratio[a] | Axial Ratio[b] | Radius, Einstein-Stokes Sphere |
|---|---|---|---|---|
| 8.55 | 1.93[c] | 1.02–1.06 | 1.8:1–2.3:1 | 19 A |
| 2.00 | 1.6 | 1.2 –1.3 | 4.4:1–5.6:1 | 23 A |

[a] 20–30% hydration. [b] 20–30% hydration, prolate and oblate ellipsoids. [c] Taken from Kronman and Andreotti (1964).

have, of course, only relative significance and should not be taken to be accurate descriptions of the physical dimensions of the molecule. The decrease in $s_{20,w}$ observed in going from *p*H 8.55 to 2.00 may be regarded as arising from an increase in the asymmetry of the molecule (considered to be an ellipsoid of revolution) from an axial ratio of about 2:1 to one of about 5:1. Alternatively, if we regard the change in frictional properties to be caused by isotropic swelling of the molecule, the change in sedimentation constant would correspond to about a 20% change in molecular radius. It is apparent that acidification of α-lactalbumin from the alkaline side of the isoelectric point to *p*H 2 yields an "expanded" molecule. Thus we can conclude that the associating unit at *p*H 2 is not the native molecule but one with a somewhat different structure.

## DISCUSSION

*State of Aggregation from pH 2 to 6.*—Because of the variety or observations made in this *p*H region it seems worthwhile to briefly summarize them. The results presented here and in the previous paper (Kronman and Andreotti, 1964) clearly indicate the marked propensity of α-lactalbumin to association or aggregation. The extent to which this occurs, as well as the manner in which it is manifested, depends strongly on *p*H.

In the *p*H region below 4 both ultracentrifuge and solubility measurements show a time-dependent aggregation process yielding polymers whose molecular weights are probably in excess of $3 \times 10^5$. In this same *p*H region sedimentation-equilibrium and velocity measurements show that association to low-molecular-weight species also occurs (Kronman and Andreotti, 1964). Close to *p*H 4 the rate of aggregation decreases, and precipitation now occurs and continues until about *p*H 5.25. This precipitate appears to be distinguishable from the soluble aggregate formed at

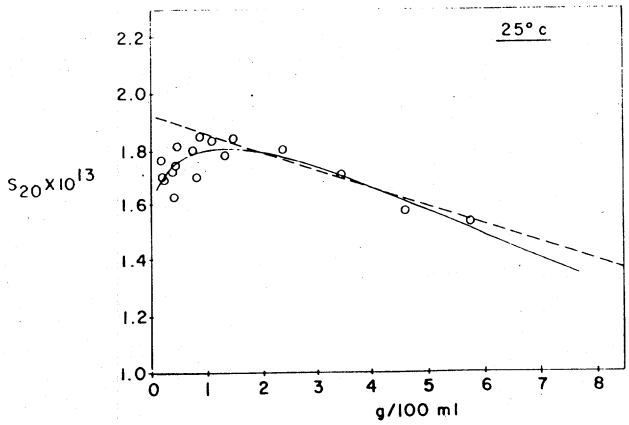


FIG. 5.—Concentration dependence of the slow component of α-lactalbumin at *p*H 2.00, 0.15 M KCl, 25.0°. Preparation 3088. – – – –, *p*H 8.55.

lower *p*H values on the basis of the rates of solubilization at *p*H 6.6.

On the alkaline side of the isoelectric zone (*p*H 5.24–6.00), while association is observed, it is considerably weaker than on the acid side (Kronman and Andreotti, 1964); time-dependent aggregation is absent, assymetry of ultracentrifuge patterns occurring only at very high protein concentrations. Since our primary interest in these studies has been the behavior of α-lactalbumin in acid solution, we have not carried out extensive measurements on the alkaline side of the isoelectric point and do not know to what extent association persists at *p*H values higher than 6. It is interesting to note, however, that the slope of the $\bar{M}_w$ versus *c* curve at *p*H 8.55 (Fig. 1, Kronman and Andreotti, 1964) is positive, suggesting that attractive interactions occur even at this relatively high *p*H. It is apparent from these data that α-lactalbumin does not exist in monomeric form in the *p*H range 2–6 except at very low concentration and low temperature on the alkaline side of the isoelectric point.

*Association and Aggregation below pH 4.*—The manner in which we have distinguished "association" (Kronman and Andreotti, 1964) from "aggregation" implies a distinction between the two processes. We have assumed for the sake of convenience that the presence of two boundaries in the ultracentrifuge implied two distributions of components corresponding to two processes:

(1) association:

$$2\,A \rightleftharpoons A_2; \quad 3\,A \rightleftharpoons A_3 \ldots fA \rightleftharpoons A_f$$

(2) aggregation:

$$eA \rightleftharpoons A_e; \quad mA \rightleftharpoons A_m \ldots nA \rightleftharpoons A_n$$

where A represents the monomeric α-lactalbumin molecule and $A_i$ the various polymers with $f << e < n$. This scheme assumes that polymers intermediate in size between $A_f$ and $A_e$ are absent. While the experimental results may be consistent with such a scheme, they do not exclude the possibilities of one such as:

$$2\,A \rightleftharpoons A_2; \quad 3\,A \rightleftharpoons A_3 \ldots nA \rightleftharpoons A_n$$

This series of consecutive reactions, in contrast with the pair of parallel reactions, involves all polymers including those of intermediate size. The ambiguity in the interpretation of the sedimentation-velocity measurements arises from the fact that the number of boundaries resolved in associating systems will depend upon the rates of reaction and reversal as compared to

the rate of sedimentation. This problem has been discussed in great detail by Fujita (1962) and will receive only cursory consideration here. In such reaction systems two extreme cases can be distinguished.

(1) Where the rates of association and dissociation are very slow, the schlieren pattern will describe the component distribution anticipated from the stoichiometry and equilibrium constant for the reaction. In effect the system will behave as a mixture of noninteracting components.

(2) Where the rates of association and dissociation are very rapid, the component distribution seen in the ultracentrifuge will *not* represent the actual composition of the reaction mixture. In spite of this lack of correspondence between component distribution and composition, such systems can often be described quantitatively. For example, Timasheff and Townend (1961) have been very successful in applying the theory of Gilbert (1959) to a sedimentation-velocity study of the association of $\beta$-lactoglobulin.

The association of $\alpha$-lactalbumin at *p*H 3.00 at very low concentrations appears to be an example of the latter situation (Kronman and Andreotti, 1964). Under these conditions even though aggregate is absent and direct molecular-weight measurements indicate association, a single boundary is observed in sedimentation-velocity experiments. This is not surprising in view of the fact that the rates of association and dissociation were found to be relatively rapid (Fig. 4, Kronman and Andreotti, 1964).

On the other hand, the rates of aggregation and disaggregation of high-molecular-weight polymers are rather slow. We might anticipate that formation and dissociation of polymers of intermediate size would occur at a rate intermediate between that for fast and slow "components." In such a case we would have a continuous change in rates of association and dissociation from rapid to slow. Under such conditions it is difficult to imagine how the component distribution in a sedimentation pattern could represent the composition of the system. The bimodel sedimentation patterns would then be solely a reflection of these differences in the rates of aggregation and disaggregation of polymers of $\alpha$-lactalbumin.

One should not be too quick to reject the view that solutions of $\alpha$-lactalbumin might consist of mixtures of low-molecular-weight associated particles and high-molecular-weight polymers with no polymers of intermediate size. Such a situation is rather typical of solutions of soaps where the distribution of micelle sizes is apt to be rather narrow. Unfortunately, with the information at our disposal at present this question cannot be answered. For much the same reason we shall not attempt to describe the stoichiometry of the low-concentration association or estimate the molecular size from the sedimentation constant of the slow component. (The problem of evaluating the molecular size of the associated unit from sedimentation-velocity data is further complicated by our ignorance as to the sedimentation constant of the monomeric unit in acid *p*H. The value approached at infinite dilution at *p*H 2 is markedly lower than that for the protein at *p*H 8.55 (Fig. 5). As we shall discuss subsequently, this decrease appears to be the consequence of a conformational change which occurs *above* *p*H 3. The sedimentation constant of monomeric $\alpha$-lactalbumin at *p*H 3 might likewise be lower than that of the native molecule.)

*Origin of the Aggregation at Acid p*H.—The mode of intermolecular interactions occurring for $\alpha$-lactalbumin at acid *p*H is radically different from that observed at *p*H values above 4. The effect of *p*H, ionic strength, and nature of electrolyte on the extent of aggregation of $\alpha$-lactalbumin all suggest that coulombic repulsion plays an important role in determining the rate of the process. Yet, on the alkaline side of the isoelectric point (*p*H 5.24–6) where the absolute value of the protonic charge is low, neither ultracentrifugation nor solubility changes in ammonium sulfate reveal aggregation comparable to that observed on the acid side. Indeed, reversal of acid aggregation can be accomplished at *p*H 6.6 (Fig. 3). Moreover, the extent of association at low concentrations of protein is greater at *p*H 3 than at 5.24 and at 6 with a clear-cut temperature coefficient being observed in the case of association at low *p*H (compare Figs. 4 and 8, Kronman and Andreotti, 1964).

The increased aggregability of $\alpha$-lactalbumin at acid *p*H as compared to *p*H 5.24 or 6.00 cannot be the result of a gross reduction of net charge resulting from preferential anion binding. For the charge at *p*H 2 to be less than or equal to that at *p*H 6.00, more than 77 moles of chloride ion/$10^5$ g of protein would have to be bound in excess of that at *p*H 6.00.[1] This is considerably larger than values typically obtained for proteins; e.g., bovine serum albumin, a protein having great affinity for anions, binds only 45 moles of chloride ion/$10^5$ g of protein (Carr, 1952).

It would thus appear that the attractive forces between $\alpha$-lactalbumin molecules on the acid side of the isoelectric zone are considerably larger than those in the *p*H region 4–6. These attractive forces must be sufficiently large so that *small* reductions in net charge due to ion binding or reduction of electrostatic repulsion by electrolyte screening are sufficient to produce extensive aggregation or precipitation.

This hypothesis of increasing intermolecular attractive forces on the acid side of the isoelectric point implies that the state or distribution of amino acid side chains at the molecular surface has been altered. Such changes with *p*H could come about in several ways.

(1) Changes in the states of ionization of certain key groups: This condition could manifest itself in a variety of ways ranging from formation of salt bridges between acidic and basic groups to the electrostatic attraction operation between identical molecules owing to fluctuations in charge and charge configuration (Kirkwood and Shumaker, 1952). Aside from these purely electrostatic effects, one might anticipate that ionization of certain strategically placed groups would modify interactions occurring through hydrophobic portions of the molecular surface.

(2) Alteration of molecular conformation resulting in change in the surface density of hydrophobic and hydrophilic groups: The decreased solubility of denatured protein is the most common example of this. The conformational change, however, need not be as extensive as that encountered in denaturation. For example, the limited conformational change occurring during the N-F transition of bovine serum albumin at acid *p*H yields a molecule with decreased solubility (Rachinsky and Foster, 1957). Such a process may, of course, be regarded as a kind of reversible denaturation.

The experimental evidence presented here and in collateral studies indicates that the aggregation in acid *p*H is paralleled by a conformational change in the $\alpha$-lactalbumin molecule. Thus, while other explanations of the acid-*p*H aggregation may not necessarily be ruled out, it does not seem necessary to invoke them.

[1] This was calculated from protonic charges computed from the amino acid data of Gordon and Ziegler (1955), using a *pK* of 4.6 (Tanford, 1963) for carboxyl groups.

*Conformational Changes of α-Lactalbumin.*—The monomeric α-lactalbumin molecule at *p*H 2 exhibits a frictional ratio higher than that of the native molecule (Table V). This increase in frictional ratio is the consequence of a change in conformation occurring in acid *p*H. Other evidence for such a conformational change has been obtained by differential spectral measurements in the tyrosine-tryptophan region (270–300 mμ) and in the 230-mμ region (M. J. Kronman, L. Cerenkowski, and L. Holmes, paper in preparation), by rotatory dispersion measurements (M. J. Kronman, R. Blum, and L. Holmes, unpublished data), and by fluorescence measurements (M. J. Kronman, paper in preparation). Since these measurements will be described in subsequent publications they will be given only cursory consideration here.

Spectral studies (M. J. Kronman, L. Cerenkowski, and L. Holmes, paper in preparation) show a differential ultraviolet spectrum of acidified α-lactalbumin (using *p*H 5.8–6 protein as a reference) characteristic of alterations in the environment of tryptophan (Wetlaufer, 1963).

The relationship of this conformational change to the aggregation can be seen by a comparison of the *p*H dependence of the aggregation to that for the conformational change. This is shown in Figure 6. The per cent change in conformation was taken to be $100[\Delta E/(\Delta E)_{max}]$, where $(\Delta E)_{max}$ is the maximum differential extinction coefficient at 293 mμ obtained at low *p*H. Shown also is the curve for the ammonium sulfate solubility data taken from Figure 4. About 75% of the conformational change occurs in the *p*H region 3.5–4. In this same *p*H region the extent of aggregation falls off, in spite of the decreasing molecular charge.

It seems difficult to escape the conclusion that aggregation observed for α-lactalbumin at acid *p*H is the consequence of a "denaturationlike" conformational change which alters the nature of groups at the molecular surface. The overall process can be represented by:

$$A_n \rightleftharpoons A_d \text{ (denaturation)}$$

$$nA_d \rightleftharpoons (A_d)_n \text{ (polymerization)}$$

For the sake of simplicity we have included low concentration association with aggregation as the "polymerization" step. The fact that association appears to be much stronger on the acid side of the isoelectric point leads us to believe that this too is a consequence of the conformational change.

The conformational change reflected as changes in rotatory dispersion, differential spectra, and fluorescent intensity occurs quite rapidly with the extent of the transformation at *p*H 3.0 being only slightly less than that at *p*H 2.00 (Fig. 6). In contrast with this the aggregation is markedly time dependent, the rate being much slower at *p*H 2 than at *p*H 3. Thus the actual aggregation of denatured α-lactalbumin must be the rate-determining step. This conclusion is lent support by the observation that the rate of insolubilization does not follow first-order kinetics.

Since the aggregation is the rate-limiting step in the overall denaturation process, the effect of anions such as nitrate must be the promotion of aggregation through reduction of net charge on the protein molecule. This is in contrast with the observations of Metzger and Edelhoch (1961), who showed that for thyroglobulin the rate of *denaturation* itself was a function of anion species.

Zittle (1956) has suggested that the changes in solubility of α-lactalbumin occurring on addition of salt at alkaline *p*H (the so-called *solubility transformation*)

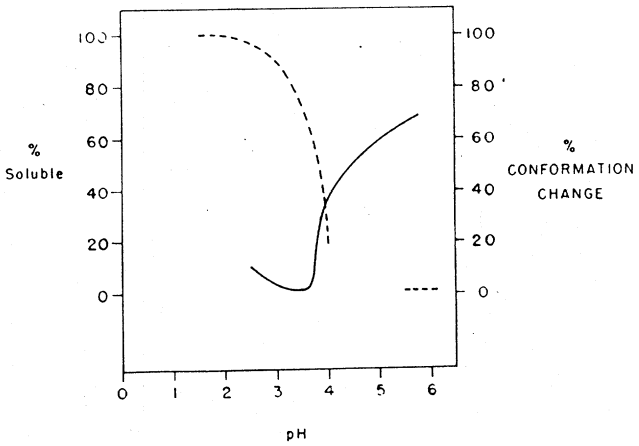


FIG. 6.—Comparison of *p*H dependence of aggregation and conformational change. ———, solubility data from Fig. 8; – – – –, per cent change in conformation calculated from differential spectral data (M. J. Kronman, L. Cerenkowski, and L. Holmes, in preparation). Temperature, 25.0°; 0.15 M KCl.

were the consequence of a change in physical configuration of the molecule on binding of anions. It would appear from our studies that the change in conformation actually occurs at *p*H values just acid to the isoelectric point. In view of the results presented here we are inclined to identify their less-soluble component with the aggregate formed at *p*H 4 and below. The relative slowness of reversal even at *p*H 6.6 (Fig. 3) would make it possible to "freeze in" aggregation occurring in the region of *p*H 4 during the course of preparation of the protein and subsequent steps such as lyophilization. The solubility transformation described by Zittle (1956) would then correspond to reversal of an aggregation occurring at lower *p*H.

*Solubility of α-Lactalbumin.*—While it seems clear that the aggregation occurring in acid *p*H is the consequence of a "denaturationlike" conformational change, occurring largely below *p*H 4, it seems more than coincidental that α-lactalbumin exhibits characteristically low solubility at *p*H values just above this—the isoelectric region, *p*H 4.25–4.50. The molecular characteristics of the native protein are such that this low solubility is rather unexpected. The frictional ratio of the native molecule (Table V) is nearly unity, indicating a compact nearly spherical molecule. The frequency of nonpolar side chains, 0.33, does not appear to be out of the ordinary when compared with that for other globular proteins such as β-lactoglobulin (0.32) or human serum albumin (0.37) (Waugh, 1954).[2]

The solubility of α-lactalbumin, or for that matter any protein, will be determined by the densities of nonpolar and polar side chains at the molecular surface. At present we cannot say for α-lactalbumin whether this is the consequence of a particularly favorable distribution of groups in the native state or an alteration of such a distribution as the protein is titrated to the region of its isoelectric point. Both explanations seem equally reasonable at present. Insulin, for example, shows low isoelectric solubility and association and fibril formation on the acid side of the isoelectric point with no apparent conformation change (Waugh, 1954). Yet, the fact

[2] The frequency of nonpolar chains as defined by Waugh (1954) is equal to the number of such chains (valine, leucine, isoleucine, proline, phenylalanine, tryptophan, and tyrosine) divided by the total number of side chains. The amino acid data of Gordon and Ziegler (1955) was utilized in the calculation.

that the conformational change observed in α-lactalbumin occurs *just* below the isoelectric region makes it difficult to reject the view that a subtle alteration of the molecular surface of the protein occurs in the isoelectric region. Clearly, further study of the isoelectric protein will be required to answer this question.

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# Metabolites of *p*-Aminobenzoic Acid. IV. Structure of "Metabolite I" and Aryl Hydroxylation Prior Hydroxymethylation of the Benzene Ring*


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The previously designated, biologically active *p*-aminobenzoic acid metabolite I ($C_{28}H_{30}N_4O$) is not a metabolite, but rather, is chemically synthesized from four *p*-aminobenzyl alcohol moieties that are formed by the direct enzymatic reduction of *p*-aminobenzoic acid. The structure of the tetramer is proved to be *N*-{*N*-[*N*-(*p*-aminobenzyl)-*p*-aminobenzyl]-*p*-aminobenzyl}-*p*-aminobenzyl alcohol by chemical and proton nuclear magnetic resonance studies. The presence of a hydroxymethyl group in the tetramer and the metabolic substrate role of *p*-aminobenzyl alcohol suggested that aniline hydroxylation may be preceded by prior hydroxymethylation. This intermediate step is verified and the following new metabolic pathway is elucidated: aniline → *p*-aminobenzyl alcohol → *p*-hydroxyaniline.


Investigations of the metabolism of *p*-aminobenzoic acid by acid-fast bacteria resulted in the isolation of *p*-hydroxyaniline, aniline (trace amounts), and two crystalline compounds previously designated metabolites I and II (Sloane *et al.*, 1951, 1954; Sloane, 1961). All the above compounds accumulated in the medium after the metabolism of added *p*-aminobenzoic acid. Metabolite II was identified as *p*-aminobenzyl alcohol (Sloane and Untch, 1962). Further metabolism of this compound, which is formed by enzymatic reduction of *p*-aminobenzoic acid, resulted in the formation of *p*-hydroxyaniline (Sloane *et al.*, 1963). It has now been shown that the biologically active, previously designated metabolite I, hereafter called compound I, is not a metabolite, but rather is chemically synthesized from the metabolic product, *p*-aminobenzyl alcohol. The occurrence of compound I in all fermentation experiments with *p*-aminobenzoic acid was therefore caused by chemical synthesis that occurred during the isolation procedures employed.

In this paper we determine the structure of compound I, suggest a possible role for this compound in aniline hydroxylation, and prove that enzymatic hydroxylation of aniline is preceded by hydroxymethylation of the benzene ring.


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## Results and Discussion

The degradation products of compound I and substrate used for biological studies reported in this paper originated from synthetic compound I. Synthetically prepared compound I was proved to be identical with the material ("metabolite I") previously isolated from fermentation experiments with *p*-aminobenzoic acid by superimposable infrared and ultraviolet spectra, melting behavior, and biological activity (Sloane, 1961). Compound I is spontaneously deposited as crystalline material from a weakly acidic buffer solution of *p*-aminobenzyl alcohol. Initially, compound I was crystallized from methanol to give micro crystals, mp 198–199° (Sloane, 1961). Later, it was found that benzene is a better solvent for recrystallization. The melting point of compound I is not discrete and depends upon the conditions employed when making the determination.[1]

*Identification of Isolated Degradation Products of Compound I.*—Treatment of compound I with 8 N

[1] The following observations were made using evacuated capillary tubes: preheated bath (140°) mp 238–242°; preheated bath (150°), transition 152°, mp 238–241°; preheated bath (170°), immediate melting, gas evolution with resolidification, mp 240–242.5°.